

Mini Review

TRANSGENIC PLANTS EXPRESSING *BACILLUS THURINGIENSIS* DELTA-ENDOTOXINS

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Abstract Commercial varieties of transgenic *Bacillus thuringiensis* (Bt) plants have been developed in many countries to control target pests. Initially , the expression of native Bt genes in plants was low due to mRNA instability , improper splicing , and post-translation modifications. Subsequently , modifications of the native Bt genes greatly enhanced expression levels. This is a review of the developments that made modern high-expression transgenic Bt plants possible , with an emphasis on the reasons for the low-level expression of native Bt genes in plant systems , and the techniques that have been used to improve plant expression of Bt toxin genes.

Key words *Bacillus thuringiensis* , Cry protein , genetic modification , insecticidal protein , transgenic plants

1 INTRODUCTION

The impact of crop damage caused by pests is staggering worldwide , despite the applications of sophisticated crop protection measures , such as synthetic pesticides. The costs associated with management practices and chemical control of pests approach \$ 10 billion annually. However , global loss from insects is still 20% to 30% of total crop production (Oerke 1994 , Estruch *et al.* 1997).

Integrated pest management is plagued with problems , such as environmental contamination and pesticide control failures. The number of insects that are resistant to synthetic insecticides is increasing and resistance to pesticides is recognized as a serious threat to integrated pest management. To date , at least 540 insect and mite species have developed resistance to at least one synthetic insecticide , with many examples of cross-resistance (Clark

and Yamaguchi 2002). An extreme example is the Colorado potato beetle , *Leptinotarsa decemlineata* , which can no longer be controlled with synthetic insecticides in some regions of the United States (Ferro and Boiteau 1992).

To address some of the shortcomings associated with synthetic pesticides , researchers have developed and farmers have been using genetic engineering to improve crop resistance to insect damage. Advantages of transgenic crops include highly efficient control of target pests with reduced environmental pollution. Over the past 15 years , the success in producing insect-resistant crops through the transfer of insecticidal protein genes has been impressive. A milestone was reached in 1995 , when the first generation of insecticidal plants , known as Bt-corn , Bt-cotton , and Bt-potato , were registered by the United States Environmental Protection Agency (US EPA) for commercial application. To

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date, twelve countries have permitted the commercial production of Bt crops, cultivated on 11.4 million ha worldwide in 2000 (Shelton *et al.* 2002). Bt, which had limited use as a foliar-spray insecticide, has become a major insecticide. Moreover, many other transgenic plants expressing insect-resistant proteins are under development through the incorporation of Bt and non-Bt genes. Bt and other transgenic technology are shaping the future of agriculture.

Non-Bt insect-resistant proteins include lectins (Chrispeels and Raikhel 1991), cholesterol oxidases (Linder and Bernheimer 1984, Purcell *et al.* 1993), chitinases (Ding *et al.* 1995, Kramer and Muthukrishnan 1997), avidin (Kramer *et al.* 2000), and a variety of proteins that interfere with the nutritional needs of insects, such as proteinase inhibitors (Hilder *et al.* 1987, Oppert 2000), α -amylase inhibitors (Huesing *et al.* 1991), and polyphenol oxidases (Felton *et al.* 1992). Some of these insect-resistant protein-encoding genes have been cloned and engineered into plants (Johnson *et al.* 1990, White *et al.* 1993). However, successful commercial application has not been achieved because of low efficacy and/or other difficulties.

In this mini review, the Bt proteins and the technology associated with transgenic Bt plants are discussed, with a focus on the causes of low-level expression of native Bt genes in plant systems and techniques used to improve expression. The benefits and risks associated with Bt technology are also discussed.

2 BT CRY PROTEINS AND MODE OF ACTION

Bt is a group of gram-positive, spore-forming, insect-pathogenic bacteria, initially isolated in Japan by Ishiwata and formally described by Berliner in 1915 (Berliner 1915, Perferoen 1997). It is characterized by producing insecticidal crystalline inclusions during sporulation (Hannay 1953). The crystalline inclusion is composed of δ -endotoxins, or crystal (Cry) proteins (Höfte *et al.* 1986). Cry proteins were originally classified based on their tar-

get insect range and primary structure, including lepidopteran- (Cry1, about 130 kDa in molecular mass), lepidopteran- and dipteran- (Cry2, 68–71 kDa), coleopteran- (Cry3, 73 kDa), and dipteran-active proteins (Cry4, 72–135 kDa) (Höfte and Whiteley 1989). However, confusion in the original classification system and ongoing discovery of Cry proteins with very different amino acid sequences and insecticidal activities necessitated the development of a new classification system. The new system groups Cry proteins based on their amino acid or cry gene sequences only (Crickmore *et al.* 1998). Presently there are 40 different groups, with a number of members in each group (www.bio.susx.ac.uk/home/Neil_Crickmore/Bt). Inactive Cry proteins (protoxins) are usually incorporated into the crystals or parasporal bodies.

The primary targets of Bt toxin are cells in the brush border membrane of the insect midgut. In general, the mode of action of Cry proteins involves solubilization of the crystals in the susceptible insect midgut, proteolytic processing of the protoxin by midgut proteinases, binding of the activated toxin to midgut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores (Gill *et al.* 1992, Rajamohan *et al.* 1998). In a lepidopteran insect, Bt crystals are solubilized in the alkaline conditions of the gut, releasing Bt protoxin (Knowles and Dow 1993). Once solubilized, protoxins are processed by digestive proteinases, such as trypsin and chymotrypsin proteinases. The 130- to 135-kDa protoxins are hydrolyzed progressively from the C-terminus toward the N-terminus, with a minor digestion of the N-terminus, resulting in a 55- to 65-kDa proteinase-resistant toxic core (Höfte and Whiteley 1989). The C-terminus is not essential for insect toxicity and contains a high level of cysteine amino acid residues. This region has been proposed to be necessary for crystallization of the protoxin and protection of the toxin from premature proteinase cleavage (Adang *et al.* 1985, Höfte *et al.* 1986). Some Cry proteins, such as Cry3A lack the C-terminal domains and undergo some proteolytic processing at the N-terminus (McPherson *et*

al. 1988). The activated molecules traverse the peritrophic membrane and bind specifically to receptor proteins on the epithelial membrane , such as cadherin and aminopeptidase N (APN) (Ferré *et al.* 1991 , Knight *et al.* 1994 , Vadlamudi *et al.* 1995). Once the toxin molecules bind to receptors , a conformational change produces a pore in the membrane . Several toxin molecules form a non-specific pore that allows a large influx of K^+ ions and efflux of H^+ ions (Knowles and Dow 1993). This process results in a higher osmotic concentration in the cells , which leads to absorption of water into the gut cells , cell swelling and cell lysis .

3 TRANSFORMATION OF PLANTS WITH BT *cry* GENES

Most microbial Bt isolates have several extra-chromosomal elements and the Bt Cry proteins are generally encoded by large plasmids (Gonzalez *et al.* 1982). These extra-chromosomal elements are responsible for the high level of diversity of Cry proteins in Bt strains . The first crystal protein genes were fully sequenced in 1985 (Adang *et al.* 1985 , Schnepf *et al.* 1985). Presently , more than 100 *cry* genes have been cloned and sequenced from various Bt strains (Rajamohan *et al.* 1998). The impetus to clone new Bt Cry genes is associated with the great potential that they have for the commercial development of transgenic insect-resistant plants .

The finding of plasmids in another soil-born bacterium , *Agrobacterium tumefaciens* , initiated and promoted the development of transgenic plant technology . The Bt genes *cry1Aa* , *cry1Ab* or *cry1Ac* , encoding δ -endotoxins isolated from the lepidopteran-active *Bt* subsp. *kurstaki* , were first introduced into tobacco (Adang *et al.* 1987 , Barton *et al.* 1987 , Vaeck *et al.* 1987) , tomato (Fischhoff *et al.* 1987) and potato (Gasser and Fraley 1989) by *Agrobacterium*-mediated transformation with different promoters . Their expression conferred some degree of protection against the tobacco hornworm , *Manduca sexta* , tobacco budworm , *Heliothis virescens* , tobacco bollworm , *Helicoverpa zea* , and potato tuberworm , *Phthorimaea operculel-*

la . *Agrobacterium*-mediated transformation was usually effective only for dicot plants , but not for monocot plants (Klee *et al.* 1987). New transformation techniques were developed to enhance transformation efficiency , including polyethylene glycol- , electroporation- , and microprojectile bombardment-mediated transformations (Potrykus 1991). Microprojectile bombardment has been most preferred by scientists due to its effectiveness on monocots and its convenience . This technique has been widely employed to transfer foreign genes into a variety of tissues and cell lines .

4 EXPRESSION OF NATIVE *cry* GENES IN PLANTS

Following transformation , a critical factor required for activity is the proper levels of expression of the insecticidal gene in plant cells . Expression levels of native *cry* genes in Bt cells are generally high , accounting for 20% to 30% of the dry weight of sporulated cells (Schnepf *et al.* 1998). However , in early transformation experiments with tobacco , tomato and potato , the expression of native *cry* genes in plants was very low (a few ngs per mg of plant protein) , and generally not sufficient to provide adequate field protection to relevant pests (Estruch *et al.* 1997).

A major reason for the low-level expression of Cry protein was that *cry* gene mRNA transcript was highly unstable and degraded rapidly (Murray *et al.* 1991). The steady-state levels of mRNA were about 100-fold lower than RNA levels from the flanking reporter gene when detected with northern blots (Adang *et al.* 1993). The reasons for the mRNA instability were extensively studied and revealed a number of problems . First , there was a difference in the composition of DNA between bacterial *cry* genes and typical plant genes , and this difference was associated with the instability of the mRNA (Perlak *et al.* 1991 , Adang *et al.* 1993). Usually , *cry* genes are more A/T rich than plant genes . For instance , A/T content of the coding region of native *cry1Ab* and *cry3A* genes is 63%—64% , about 10% higher than that found in a typi-

cal plant gene's coding region. More importantly, these A/T-rich regions resemble plant introns or potential plant polyadenylation signal sequences (ATTTA), which may cause improper processing and premature termination of the translation (Murray *et al.* 1991). Premature termination of translation induces a rapid degradation of the target mRNA. Secondly, different preferences in codon usage by plant and bacterial systems resulted in inefficient translation (Murray *et al.* 1989). For example, to code for the amino acid glutamate, there are two codons available, GAG and GAA. Monocots usually use the GAG codon (accounting for 75% of the total codon usage), while dicots do not have this preference. However, Bt bacteria prefer the GAA codon (75%). The native *cry* gene containing GAA, therefore, is not effectively expressed in a monocot plant system. Additionally, regulatory factors, such as promoters and flanking sequences, may be significantly involved in expression efficiency in plant systems (Koziel *et al.* 1996).

5 MODIFICATIONS AND EXPRESSION OF *cry* GENES IN PLANTS

To solve these problems and increase the expression of Cry toxins in plants, several approaches were proposed. The first approach was the modification of native *cry* gene sequences by increasing their G/C content, choosing plant preferred codon patterns, and eliminating sequences that might cause mRNA destabilization and splicing (Perlak *et al.* 1990, 1991, 1993, Adang *et al.* 1993). All modified genes expressing Cry proteins were carefully screened for retention of insecticidal activity. Several laboratories constructed *cry* genes modified to various degrees that resulted in significant improvements in the expression of Cry proteins in cotton (Perlak *et al.* 1990), tomato (Perlak *et al.* 1991), and potato (Perlak *et al.* 1993, Adang *et al.* 1993). Synthetic *cry* genes were essential to express Cry toxins in monocots such as maize (Koziel *et al.* 1993).

Perlak *et al.* (1991) examined several versions of modified *cry1Ab* and *cry1Ac* genes in both

transgenic tobacco and tomato and analyzed the increased expression associated with various sequence modifications. All genes were under the control of a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer region. Two types of modified genes were used. One was partially modified, while the second was fully modified. The partially modified *cry1Ab* gene had 62 of 1743 bases changed to eliminate regions with potential polyadenylation signals and A/T-rich regions. The partially modified *cry1Ab* gene had 97% homology with the native gene and a G/C content of 41% increased from 37% in the native *cry1Ab* gene. The fully modified *cry1Ab* gene had 390 of 1845 bases changed to remove all ATTAA sequences and regions of potential mRNA secondary structure, and also replaced bacterial codons with plant-preferred codons. The fully modified gene had 79% homology with the native gene, and its G/C content was increased from 37% to 49%. Most transgenic tomato and tobacco plants expressing the partially modified gene produced Cry1Ab endotoxin at levels of 1–200 ng Cry1Ab per mg of total protein. Over 10% of the fully modified *cry1Ac* and *cry1Ab* transgenic tomato and tobacco plants had expression levels in the range of 600–2 000 ng of Cry protein per mg of total protein. Compared with the native truncated gene, the highest expressing transgenic plants containing a partially and fully modified gene increased expression by 10- and 100-fold, respectively.

Similarly, expression levels of Cry3A were improved using synthetic *cry3A* genes. Adang *et al.* (1993) synthesized a *cry3A* gene and examined its expression in carrot and maize protoplasts, as well as in transgenic potato plants. The codon pattern of this synthetic gene was altered to match the codon pattern of a dicot. The G/C content of the modified *cry3A* gene in this study was 45%, altered from 36% in the native Bt gene. The synthetic gene produced the expected size of mRNA and Cry3A protein in electroporated carrot or maize protoplasts. The levels of Cry3A obtained in carrot and maize protoplasts were similar, 0.001% to 0.005%, or

10 to 50 ng/mg total protein. The native gene did not produce detectable Cry3A. Transgenic potato plants containing the synthetic *cry3A* gene were estimated to produce Cry3A at levels up to 0.025% of total protein.

The first example of a cereal plant expressing a Cry toxin was created with a synthetic gene (Koziel *et al.* 1993). A synthetic gene encoding the first 648 of the 1 155 amino acids in Cry1Ab protein produced by *Bt* subsp. *kurstaki* HD-1 was constructed using the preferred codon from maize. The synthetic gene was 65% similar to the native gene, with a G/C content of 65% compared to 37% in the native gene. Transgenic maize plants containing the synthetic gene under the control of the maize PEP-carboxylase and pollen-specific promoters produced up to 4 000 ng of Cry1Ab per mg of soluble protein in some plants. Since native *cry* genes were not expressed in maize, the magnitude of increase could not be calculated.

In addition to the modifications of coding sequences, the flanking sequences of *cry* genes were also important for their proper expression. The 5'-untranslated leader sequence (5'-UTL) of *cry* genes has been demonstrated to be significantly involved in translation efficiency. Several studies have shown that the 5'-UTL sequence, and the sequences directly surrounding the AUG start codon, can have a substantial effect on translation efficiency in both animal and plant systems (Lutcke *et al.* 1987, Wong *et al.* 1992, Koziel *et al.* 1993). In plants, a consensus sequence, UAAACAAUGGCU, was found to be important for translation efficiency (Lutcke *et al.* 1987, Koziel *et al.* 1993). In plants the preference for a guanine at the fourth residue (+4) in the gene sequence is significantly greater (85%) than in animals (38%). The preference for G in position +4 (85%) and C at +5 (77%) in plants resembles the preference for A at -3 (80%) in animals (Lutcke *et al.* 1987). In plants, the residue at the -3 position does not appear to affect translation efficiency. The CaMV 35S promoter was commonly used for transgenes and was effective for expression

of Bt genes in plants. Wong *et al.* (1992) achieved a 10–20-fold increase in Cry1Ac expression in tobacco when an *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase small subunit promoter was combined with modified 5'-UTL and chloroplast transit peptide to express a modified truncated *cry1Ac* gene in tobacco plants, compared to the CaMV 35S promoter with a double enhancer. The increase in expression was the result of a combined effect that was due to both the 5'-UTL and the transit peptide.

Ingelbrecht *et al.* (1989) found that the 3'-end regions of transgenes in plants had an effect on their expression. Neomycin phosphotransferase II (*NptII*) was used as the reporter gene, and the CaMV 35S was used as the promoter. An *NptII* gene lacking plant 3'-end sequences could be expressed in transgenic tobacco, but at a level about 12-fold lower and much more variable than a comparable construct containing the 3'-end of the octopine synthase (OCS) gene. Based on these data, most transgenic Bt plants have the OCS gene fused at the 3'-end to optimize *cry* gene expression. However, more research is needed on the precise role of 3'-end sequences and their optimization for maximal expression in transgenic plants.

Other approaches to increase *cry* gene expression levels in plants include the introduction of introns into transgenes and the transformation of chloroplasts. In the first report that introns could enhance gene expression in transgenic monocot cells, the maize alcohol dehydrogenase I (*Adhl*) gene was used (Callis *et al.* 1987). When a genomic clone of the *Adhl* gene with its endogenous promoter was used to transform maize cells, the expression of the Adh protein was about 100-fold higher than if the coding region came from cDNA clones. Others have also reported that introns induced into the transcribed portion of a gene increased heterologous gene expression in plants and animals (Borenstein *et al.* 1987, Vasil *et al.* 1989). The mechanism of enhancement by introns to improve gene expression is unknown (Koziel *et al.* 1996).

DNA in the chloroplasts of plants contains a

high level of A/T content, which is similar to native *cry* genes. McBride *et al.* (1995) reported high levels of expression of an unmodified Bt *cry1Ac* gene in tobacco chloroplasts. The introduced Bt gene was present up to about 10 000 copies per cell in the chloroplast genome, and expressed at a high level, yielding up to 3%—5% of total leaf protein as Cry1Ac. Kota *et al.* (1999) reported an over-expression of Cry2Aa2 protein in tobacco chloroplasts, conferring resistance to plants against Bt-susceptible and -resistant insects. The transformed tobacco leaves expressed Cry2Aa2 protoxin at levels between 2% and 3% of total soluble protein, 20 to 30-fold higher than current commercial nuclear transgenic plants. At present, the limitations for this approach are the availability of a broadly applicable chloroplast transformation system and the transmissible traits only from the female parent.

6 FIELD PERFORMANCE AND BENEFITS FROM COMMERCIAL BT PLANTS

Laboratory and field experimental results, plus commercial application over several years in the United States and other countries, of transgenic Bt cotton, corn, and potato plants lead to the following general conclusions. First, these commercially released Bt plants are highly effective against target pests, and the control of pest populations is equal to or better than previous control methods (Hoffman *et al.* 1992, Warren *et al.* 1992, Koziel *et al.* 1993, Armstrong *et al.* 1995, Fischhoff 1996, Harris *et al.* 1996, Higgins *et al.* 1996, Buschman *et al.* 1997, Feldman and Stone 1997, Bacheler *et al.* 1998). Thus, they provide a substitute for synthetic pesticides commonly used to control target pests (Perlak *et al.* 2001). This high efficacy is due to the potency of Bt Cry proteins at the achieved levels of plant expression, together with the timing and localization of delivery of these proteins (Fischhoff 1996). For all target insects, Cry proteins are most effective against neonate larvae (Huang *et al.* 1999a, 2002). Transgenic plants deliver the Cry protein directly to the neonates immediately after hatching, when they are most sus-

ceptible. Cry protein-expressing plants also can be more effective than alternative management methods for certain insect pests. For example, the European corn borer, *Ostrinia nubilalis*, is difficult to control with insecticide sprays because of its boring behavior. With transgenic plants, the Cry protein is delivered to the emerging larvae as they consume leaves at their most sensitive stage. Even after boring into the stalk, they are still exposed to the toxin expressed in stalk tissue. In addition, growing Bt plants can be more economical and provide a greater economic return than other pest control practices, especially in the presence of high pest density. In 1999, the US EPA conducted an analysis of economic returns and insecticide reductions for commercially released Bt plants in the United States. The results indicated an overall economic benefit to growers of \$ 65.4 million (field corn), \$ 45.9 million (cotton), \$ 0.2 million (sweet corn), and \$ 0.5 million (potato), and for a total economic return of \$ 112 million (Shelton *et al.* 2002). The US EPA's analysis also indicated a reduction of 7.5 million fewer acre treatments for cotton, 0.127 million for sweet corn, and 0.089 million for potato, but did not calculate a figure for field corn because of variable insect pressure. Other benefits associated with insecticide reductions included conservation of natural enemies and non-target organisms, decreased potential of soil and water contamination, and reduced hazards for farm workers and others likely to be exposed to insecticides (Pilcher *et al.* 1997, Hilbeck *et al.* 1998, Saxena *et al.* 1999, Hellmich *et al.* 2001). Finally, commercially released Bt plants have been evaluated thoroughly by the US EPA, and no unreasonable risks to human health or to the environment have been observed. It appears likely that this biotechnology is lower in risks and greater in benefits than current alternative technologies.

7 RESISTANCE MANAGEMENT

The high efficacy of transgenic Bt plants against target pest insects increases the likelihood that pests will develop resistance to Bt toxins.

Laboratory selection experiments with Bt toxins have indicated that many insect species have the potential for resistance to Bt formulations or individual Bt toxins (McGaughey 1985, Tabashnik *et al.* 1990, Gould *et al.* 1992, Huang *et al.* 1997, 1999b, Ferré and Van Rie 2002). Resistance management for Bt plants remains a serious concern. The currently preferred resistance management strategy for Bt plants is the “high dose/refuge strategy” (Ostlie *et al.* 1997). It was proposed based on the assumptions of recessive or partially recessive Bt resistance genes and low frequency of resistant alleles in the field. A certain proportion of the corresponding non-Bt plants must be planted to serve as a refuge of Bt-susceptible insect populations. In the refuge, susceptible homozygous individuals are able to develop to adulthood. They fly into the Bt-plant field and mate with any potential resistant insects from Bt plants. If resistance is recessive, individuals of the F1 generation will be susceptible and then will not be able to survive on Bt plants because they are heterozygous. The frequency of resistant alleles can thus be suppressed. Pyramiding two dissimilar toxin genes in the same plant has also been proposed as a strategy to delay Bt resistance (Roush 1996).

8 FUTURE PROSPECTS

Pest management with transgenic Bt plants is now established in some countries, and these plants provide high levels of insect control against many important pests. In determining which additional plants might be engineered to express Bt Cry proteins, local conditions, insect pressures, and suitable Bt genes should be considered. For instance, rice is the most important grain crop in Asia and is seriously attacked by many pests. The development of Bt rice would provide immense benefit for Asian countries. Transgenic Bt rice has entered field trails and soon will be commercially available in China (Tu *et al.* 2000, Ye *et al.* 2001, Zeng *et al.* 2002). When Bt plants are planted, new strategies should be developed to integrate pest and resistance management into this new pest control method to

sustain the biotechnology. As progress is made in understanding the interaction between Bt toxins and insect receptors, it should be possible to delay or diminish the potential of Bt-resistance development. It also should be possible to extend the target range of Bt-plants through mutation or substitution of the recognition site of Bt proteins. The pace of Bt Cry protein and gene discovery and characterization continues to increase. As new genes are discovered and tested, new insecticidal activities will become available. Some of these new genes are likely to be useful in generating second- and third-generation transgenic Bt plants.

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表达苏云金杆菌内毒素的转基因植物

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为防治一些重要农业害虫,转基因 Bt 作物已在许多国家商业化种植。在发展 Bt 作物的初期,未经改造的 Bt 基因被直接用来转化作物。但由于 Bt mRNA 的不稳定,不适当的剪切以及译后变异,Bt 在作物上的表达水平往往很低且不稳定。后来,科学工作者对 Bt 基因进行了一系列针对性的改造或人工合成,从而使其在植物细胞中得到高效表达。本文着重总结了这一转基因技术的发展过程。其内容包括未经改造的 Bt 基因在植物中表达低的原因以及改善 Bt 毒蛋白表达的有关技术。

关键词 苏云金杆菌 晶体毒蛋白 遗传改造 杀虫蛋白 转基因植物